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13. ABSTRACT (Maximum 200 Words)

Clinical studies indicate that high breast tumor levels of plasminogen activator inhibitor 1 (PAI-1) are associated with an increased metastatic risk, decreased survival, tumor angiogenesis and overall poor prognosis. Since PAI-1 is required for tumor angiogenesis and inhibition of capillary regression, a targeted genetic approach was used to ablate PAI-1 synthesis in endothelial cells employing antisense PAI-1 and dominant-negative constructs. Such targeting provided proof of principle that reduced PAI-1 synthesis inhibited capillary network formation by immortalized endothelial cells. Transfection of a dominant-negative version of USF-1, a bHLH-LZ protein important in PAI-1 transcription, also attenuated PAI-1 expression in response to angiogenic growth factors by inhibiting formation of functional USF-1 complexes on the PAI-1 promoter. Cell lines were created that were inducible for dominant-negative USF expression upon removal of doxycycline. These results suggest that combinational approaches targeting PAi-1 transcripts and its control network may form the basis for efficient breast cancer anti-angiogenic therapy.

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Introduction

Clinical studies have consistently demonstrated that high tumor levels of plasminogen activator inhibitor type-1 (PAI-1; Serpine1) are consistently associated with an increased risk for metastasis, significantly decreased patient survival and an overall poor prognosis (1,2). The role of PAI-1 as a determinant in disease progression is particularly relevant in the case of breast cancer where elevated PAI-1 expression in the primary breast carcinoma signals an aggressive angiogenic response (3-10). Indeed, PAI-1 tumor cytosol levels proved to have major prognostic value in a LOE study of >8,100 breast cancer patients enrolled in the European Cancer Consortium Study (2,8).

Tumor-initiated angiogenesis requires proteolysis of the endothelial basement membrane, migration of endothelial cells through the extracellular matrix (ECM) toward the angiogenic stimulus and continued endothelial proliferation behind the migrating front (11-13). Stimulated endothelial cell locomotion requires cycles of ECM adhesion-deadhesion and precise control of the pericellular proteolytic environment (12-14). PAI-1 functions in this process to limit plasmin generation by inhibiting the catalytic activity of urokinase plasminogen activator (uPA) (15,16) modulating, thereby, uPA-dependent ECM degradation and *in vivo* cell motility (17-19). While endothelial cell migration and capillary sprouting requires proteolysis (12,20,21), excessive protease activity prevents the coordinated assembly of endothelial cells into capillary structures highlighting the requirement for an appropriate proteolytic "balance" for a successful angiogenic response (22,23). Genetic studies *in vivo*, moreover, have implicated PAI-1 as an important regulator of this balance (24,25). Indeed, PAI-1 is expressed specifically in angiogenic "cords" and migrating endothelial cells as well as in stromal cells in direct contact with the sprouting neovessels but not in the quiescent endothelium (26-28). Most significantly, PAI-$1^{-/-}$ mice are incapable of mounting an angiogenic response either to transplanted tumors or implants of potent angiogenic growth factors (24,27,29); both tumor-associated angiogenesis and tumor invasiveness were restored by injection of PAI-1 expressing adenovirus (24,27). PAI-1 appears to promote angiogenesis specifically by inhibition of plasmin proteolysis, thus preserving an appropriate matrix scaffold for endothelial invasion as well as providing critical stability to the primitive tumor neovessels (18,24,27). Recent studies have shown that uPA-mediated plasmin generation activates MMP1 and 9 resulting in capillary regression (30). Inhibition of PAI-1 activity with neutralizing antibodies accelerates, whereas exogenous PAI-1 inhibits, capillary regression indicating that endogenous PAI-1 is the major negative regulator of this process (30). Continued PAI-1 expression by the formed capillary structures is required to maintain their stability and, in fact, to prevent regression. Our use of inducible vectors to disrupt PAI-1 synthesis, even in formed capillary structures, will be one novel approach to address the important question of whether PAI-1 targeting can have a therapeutic benefit on existing angiogenic networks. This is an important issue for the treatment of established primary tumors and their developed distant metastases.

Body of Report

Our work has focused on defining molecular controls on PAI-1 gene expression in normal and transformed cells and clarifying the role of the PAI-1 protein on cellular growth and invasive behavior. We have shown that it is possible to genetically manipulate PAI-1 synthesis in endothelial cells transfected with sense and antisense PAI-1 expression vectors (32,33). We hypothesize, based on our own preliminary and published data, as well as on work done by others (summarized in **Introduction**), that molecular targeting of PAI-1 expression can disrupt both the initial as well as the developed angiogenic response to tumor-derived stimuli. We have recently published a review article that details

our *in vitro* data in support of this contention (34). We propose that targeted attenuation of PAI-1 expression in the developing neovasculature that develops following implantation of human breast carcinoma cells into immunodeficient mice will inhibit the angiogenic response and limit subsequent tumor growth. We further suggest that human endothelial cells genetically-engineered to express inducible PAI-1 antisense transcripts may "home" to sites of active tumor-initiated angiogenesis, incorporate into the developing capillary network, and destabilize the tumor vasculature upon inducible ablation of PAI-1 synthesis. We expect that such engineered cells will ultimately serve as a therapeutic resource for inducible anti-angiogenic therapy of human breast cancer.

Based on the data summarized in the **Introduction**, our earlier working hypothesis is that genetically-induced temporal changes in the expression of PAI-1 may influence endothelial cell migration, capillary formation and/or capillary network stability. Efforts (in year 01 of this study) were devoted to confirmation that the genetic constructs (PAI-1 antisense expression vectors) developed would, in fact, result in attenuated PAI-1 synthesis when transfected into murine endothelial cells as well as when delivered to our established T2 line of rat cells. Transfection studies established that our selected rat PAI-1 mRNA coding sequence, when cloned in antisense orientation into CMV promoter-driven constructs (i.e., in the Rc/CMV expression vector backbone), effectively attenuted PAI-1 synthesis in both mouse (MS1) endothelial and rat (T2) cells. Subsequent work confirmed that one derivative (4HH) did not express detectable PAI-1 protein nor accumulate PAI-1 in the matrix. Wild-type T2 cells formed highly-branched and anastomizing networks when suspended in a complex support matrix consisting of a 3:1 mixture of Vitrogen-Matrigel. Many of these tubular processes had clearly evident lumens. Extensive sprout formation was evident at the tips of T2 branches; moreover, indicative of both invasive and differentiated compartments. PAI-$1^{-/-}$ 4HH cells, in contrast, failed to construct stable tubular structures and extensively degraded the gel matrix **(Figure 1)**. The same PAI-1 coding region antisense construct that proved effective in the Rc/CMV expression vector backbone was cloned into the pLNCX2 retroviral expression construct and introduced into MS1 and T2 cell lines. We previously reported that 4 MS1 and 2 T2 lines were successfully derived that were essentially PAI-1 null. Thus, at least within the setting of immortalized rodent endothelial lines, our targeting strategy (i.e., delivery of constitutively-expressing PAI-1 antisense constructs) has the expected outcome on gene expression and the tubulogenic phenotype.

Based on this supportive information, work in year 03 was devoted to work designed to address Task 1 of the original application.

Task 1. To assess the effect of *in vivo* delivery of PAI-1 antisense expression vectors on the angiogenic response to implanted human breast carcinomas.

a. Confirm vector transduction and PAI-1 antisense transcript expression in the developing tumor vasculature upon co-implantation of MDA-MB-231 human breast carcinoma cells and retroviral packaging cells into the mouse renal capsule.

b. Assess the ability of constitutive and inducible PAI-1 antisense transcript expression to disrupt the development and maintenance, respectively, of a tumor-dependent angiogenic response and the consequences of PAI-1 expression attenuation on tumor growth.

Important aspects of work completed in year 03 are as follows:

1. We continued our development of PAI-1 expression-disruption vectors to increase our repertoire of targeting constructs. To do this, we took advantage of our recent findings that PAI-1 transcription in both

murine and human cells requires an intact USF-1-binding E box in the proximal promoter (35). Nuclear extract immunodepletion/probe shift analyses implicated the USF family members USF-1 and USF-2, but not other closely related bHLH-LZ transcription factors with E box recognition activity (e.g., MYC, MAX), as 18-bp PAI-1 HRE-2 E box probe-binding factors . Supershift assays further confirmed that the bHLH-LZ proteins MYC, MAX, or MAD did not contribute to the formed complexes **(not shown)** but established that the PAI-1 E box construct was, in fact, a target for occupancy by USF-1 **(Figure 1C).** Factor binding specificity, moreover, was not simply a consequence of insufficient nuclear levels (or non-permissiveness under the conditions used) of other MYC family members (MYC, MAX, MAD) to recognize the 5'-CACGTG-3' motif. Control experiments indicated that each of these proteins could be detected in complexes developed using an E box probe with non-PAI-1 flanking sequences **(not shown).** E box occupancy by bHLH-LZ proteins, furthermore, usually requires the CA dinucleotide (at the $C_{-3}A_{-2}$ position) for binding (35). A mutant 18-bp construct was designed, therefore, in which the CACGTG motif was replaced with TCCGTG. Gel retardation comparison of the PAI-1 wild-type and mutant double-stranded deoxyoligonucleotide targets indicated that the CA→TC base change did, in fact, prevent formation of nuclear factor-probe complexes **(Figure 1D).** To assess the transcriptional consequences of this E box sequence disruption, the CA→TC dinucleotide substitution was incorporated into the rPAI-CAT(-764) reporter plasmid by site-directed mutagenesis. Wild-type (CACGTG) and mutant (TCCGTG) expression constructs were transfected separately, cells cultured in serum-free medium for 3 days then stimulated with TGF-β1 (1 ng/ml) for 24 hours. TGF-β1-inducible CAT activity in E box mutant transfectants was reduced significantly (>80%) compared to cultures expressing the wild-type reporter **(Figure 1E)** although both a transfected PAI-1-GFP chimera (driven by PAI-1 upstream sequences) as well as the endogenous PAI-1 gene were fully responsive to TGF-β1 stimulation **(Figure 1F-H).** These controls confirmed the competence of the host cells to activate both the endogenous and exogenous the PAI-1 promoters under the conditions used for CAT reporter assessments. An intact USF-1-binding HRE-2 site E box was required, therefore, for maximal TGF-β1 induced PAI-1 promoter-CAT reporter expression **(Figure 1E)** and the major PAI-1 E box-binding factor both in vitro and in vivo was USF-1.

2. To confirm the importance of USF-1 in PAI-1 gene control and to evaluate the usefulness of USF-1 functional disruption as an expression-ablating approach, we confirmed that a dominant-negative (A-USF) construct inhibits PAI-1 probe/nuclear factor complex formation and PAI-1 expression. Since growth factor-responsive E box sites in the PAI-1 gene are, indeed, USF-1 target sequences *in vivo* (35), it was important to determine if inhibition of USF function (i.e., DNA binding activity) would specifically affect TGF-β1-stimulated PAI-1 expression. Transfection of the dominant-negative A-USF expression vector did not attenuate TGF-β-stimulated ERK activation (36). A-USF transfection, however, completely blocked formation of USF/DNA complexes with the 18-mer HRE-2 PAI-1 probe **(Figure 2A)** as well as with the 45-mer PE2 region PAI-1 E box construct **(not shown)** and effectively reduced TGF-β-induced PAI-1 levels to that approximating quiescent controls **(Figure 2B).**

3. Earlier work clearly indicated that PAI-1 expression attenuation could be achieved at a significant levels in the established MS1 endothelial and T2 murine cell lines upon delivery of the Rc/CMV-based and pLNCX2-based constructs and the current work confirmed that similar targeting of the PAI-1 transcriptional control network with the dominant-negative A-USF vector in T2 cells (most importantly) had a similar effect. We have also successfully completed the construction of a doxycycline-inducible A-USF epithelial cell system in the HaCaT genetic background (previously described in year 02 annual report as in preparation) that will serve as a resource to identify USF-regulated genes other than PAI-1 as well as a transplantable target to evaluate induced transcriptional repression on tumor growth and angiogenesis. As a prelude to in vivo targeting, we needed to assess the ability of the Rc/CMV and pLNCX2 vectors to produce a similar down-regulation of PAI-1 expression in primary cultures of mouse

endothelial cells. To conduct an unbiased evaluation of the expression attenuation abilities of each construct in primary endothelial cells, evaluations were done using large vessel (aortic) and microvessel (fat pad) endothelial cells isolated from 40 C57Bl/6 mice for side-by-side comparisions with the immortalized MS1 and T2 cell lines. Unexpectedly, while PAI-1 was efficiently down-regulated in both the MS1 (by >75%) and T2 (by >90%) cells, consistent with the initial findings in the 4HH **(Figure 1)** and T2 **(Figure 3)** cell lines, only modest reductions (20-30%) were evident in the primary endothelial cultures (regardless of origin). It appears (although this will be confirmed in work to be done in extension year 04) that this effect is due to low transfection efficiency in the primary cells (for Rc/CMVIAP and A-USF) and significantly lower expression of the pLNCX2 construct in primary endothelial cells compared to the immortalized MS1 and T2 cell lines. Work in year 04 will consider the possibility that improved vector uptake or retroviral delivery/expression may actually take place in the in vivo setting of active angiogenesis and that the in vitro results, within the context of the primary endothelium, are not representative of the actual potential for expression modulation in vivo.

4. We have expanded our stable line of human microvessel endothelial (HMEC-1) cells that constitutively express a PAI-1-green fluorescent chimeric protein driven by 800 bp of PAI-1 promoter sequences. These cells will be infused into the tail vein of tumor-bearing mice to confirm that genetically-engineered endothelial cells can, in fact, be incorporated into the developing tumor-dependent angiogenic network in vivo **(Task 2a)** as we established (in year 02) for capillary structure incorporation in vitro.

5. The kidney capsule implantation model of breast carcinoma growth assessment proved to be unreliable. Orienting experiments indicated that controlled reproducible delivery of constant numbers of MDA-MB-231 human breast carcinoma cells into the kidney capsule was not possible due to significant leakage at the injection site and capsule rupture. This ambiguity in initial cellular "seeding" resulted in a significant disparity in tumor growth rate among implanted mice. Since tumor size and growth rate were important indicators of successful angiogenesis, we decided to investigate more quantifiable implantation sites and models. Preliminary experiments confirmed that direct implantation of breast carcinoma cells into the mammary fat pad not only represented a more physiologic orthotopic model but also proved more reproducible in assessment of tumor growth rate. Moreover, this site also allowed for the use of the syngeneic C57BL/6-PDVA carcinoma transplant system that is a significant adjunct to the current xenotransplantation nude mouse model in order to evaluate the therapeutic usefulness of PAI-1 gene targeting. These improved developments will be pursued in extension year 04 and are detailed in the appended revised Statement of Work.

Key Research Accomplishments

1. Use of a dominant-negative USF-1 construct (A-USF) effectively attenuated angiogenic growth factor-induced PAI-1 expression in T2 endothelial cells. It was established, using the sensitive mobility shift assay and a target PAI-1-specific double-stranded DNA probe, that the mechanism involved direct inhibition of endogenous USF-1 function (i.e., DNA motif binding).

2. We have completed construction of doxycycline-inducible dominant-negative USF constructs in the HaCaT cell genetic background that are suitable for in vivo expression "switching".

3. PAI-1 expression down-regulation was achieved in established endothelial cell lines (but not yet in primary endothelial cell cultures) supporting the hypothesis put forth in Task 2 of the original application that genetically engineered endothelial cells inducible for expression of PAI-1 antisense transcripts may

incorporate into the developing human breast tumor vasculature and disrupt the integrity of the supporting capillary network.

4. Preliminary experiments confirmed that direct implantation of breast carcinoma cells into the mammary fat pad not only represented a more physiologic orthotopic model but also proved more reproducible in assessment of tumor growth rate.

Reportable Outcomes

All genetically-engineered immortalized (MS1, HMEC-1, T2) cells are maintained in the laboratory of the PI and are available for distribution to investigators in the breast cancer field. Doxycycline-inducible dominant-negative USF plasmid constructs as well as the stable dominant-negative USF-1-inducible HaCaT cell line that is suitable for in vivo expression "switching" is also available for distribution. When the corresponding stable derivatives in the MCF-7, T2 and HMEC-1 backgrounds are completed they will similarly be made available to DOD investigators. The data obtained with the dominant-negative A-USF constructs was used as preliminary findings in support of a new grant application to the National Institute of General Medical Sciences. The success of PAI-1 gene targeting in the established T2 and MS1 cell lines will be used to support a revised grant application to the National Cancer Institute.

Conclusions

The present work is based largely on our continuing hypothesis that molecular targeting of PAI-1 expression in tumor-associated angiogenic vessels represents a unique gene therapy appoach that has the distinct advantages of (1) potential cell-specific construct targeting and (2) a high likelihood of success when directed to established angiogenic "beds". Our laboratory has had considerable experience in the construction and delivery of both sense and antisense PAI-1 expression vectors and, more recently, in the design of small molecule inhibitors of PAI-1 function. Targeted disruption of PAI-1 gene expression in endothelial cells incorporated into formed capillary structures constitutes an important approach to address the critical question of whether PAI-1 targeting can have a therapeutic benefit on developing and existing angiogenic networks. We envision that a multifaceted attempt to inhibit PAI-1 gene expression in both breast carcinoma cells (see **Introduction**) and in the collateral tumor vascular network would likely require cell type-specific expression modulation control. Our on-going program to develop novel approaches to manipulate PAI-1 synthesis (either directly or through its transcriptional control network) as well as new physiologically-relevant in vivo test systems has provided tools to address this important problem. Clearly, our major contribution is that we have established that PAI-1 expression modulation is possible in the involved cell types. The goals described in this funded program, in conjunction with the general scope of work ongoing in our laboratory, reflect these separate but focused efforts to utilize gene therapy approaches to maximize a positive outcome for breast cancer patients.

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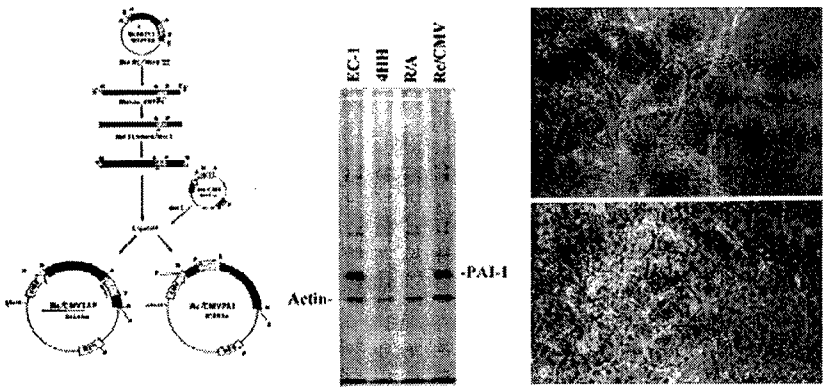

Figure 1. Targeted down-regulation of PAI-1 synthesis inhibits in vitro tubulogenesis. Rc/CMV plasmid vectors were constructed that drive expression of a full-length PAI-1 cDNA insert cloned in sense (PAI) and anti-sense (IAP) orientations, under control of a CMV promoter (**A**). The black fill-in region corresponds to the PAI-1 cDNA inset. EC-1 parental cells were transfected with the Rc/CMVIAP vector and stable clones derived. To assess the success of Rc/CMVIAP driven down-regulation of PAI-1 synthesis and matrix accumulation, saponin-extracts of 35S-methionine-labeled cells were separated by gel electrophoresis and proteins visualized by fluorography (**B**). One derivative (4HH) did not express detectable PAI-1 protein nor accumulate PAI-1 in the matrix. Cells transfected with Rc/CMV vector without insert expressed levels of PAI-1 similar to that of EC-1 controls. Wild-type T2 cells formed highly-branched and anastomizing capillary networks when suspended in a complex support matrix consisting of a 3:1 mixture of Vitrogen-Matrigel (**C**). Many of these tubular processes had clearly evident lumens. Extensive sprout formation was evident at the tips of T2 branches, moreover, indicative of both invasive and differentiated compartments. PAI-$1^{-/-}$ RHH cells (**D**), in contrast, failed to construct stable tubular structures and extensively degraded the gel matrix. Refractile bodies (**D**) are apoptotic cells.

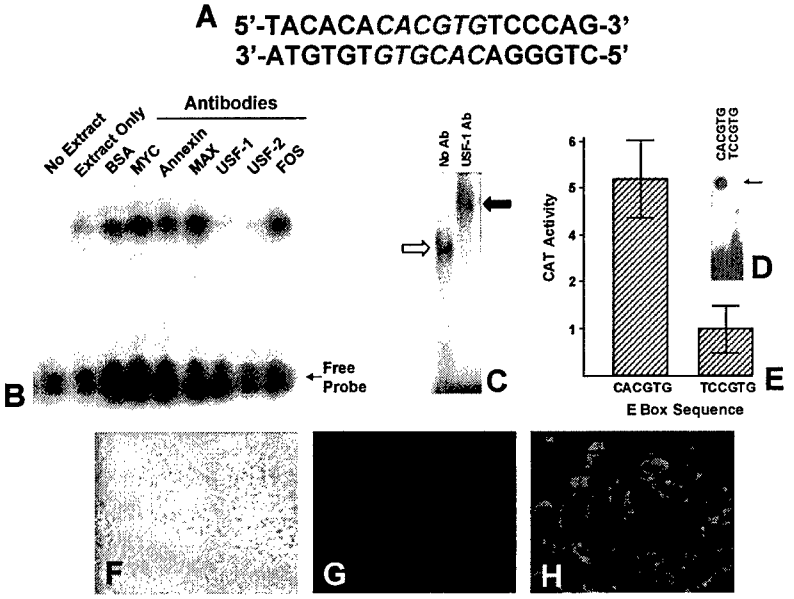

Figure 2. USF recognition of an 18-mer HRE-2 PAI-1 probe and TGF-β1-stimulated CAT reporter activity require a consensus E box (CACGTG) motif at nucleotides -160 to -165. A double-stranded 18-mer HRE-2 region PAI-1 E box probe (**A**) was end-labeled for use in nuclear immunodepletion (**B**) and gel supershift (**C**) assays. Depletion of nuclear extracts with antibodies to either USF-1 or USF-2 inhibited the formation of the characteristic band shift (**arrow**) whereas inclusion of antibodies to MYC, MAX, FOS and annexin or substitution of BSA for specific Ig were without affect (**B**). Supershift analysis confirmed that USF-1 was a major contributor to the 18-mer PAI-1 HRE-2 probe/protein complex (**C**). bHLH-LZ proteins with E box specificity usually require the CA dinucleotide for binding (White et al., 2000). A mutant 18-bp construct was designed in which the CACGTG motif was replaced with TCCGTG. Gel retardation comparison of the PAI-1 wild type and

mutant DNA targets confirmed that the CA→TC base change ablated binding of nuclear factors (**D**). Wild-type (CACGTG) and mutant (TCCGTG) PAI-1 promoter-driven CAT reporters were transfected separately, cells cultured in serum-free medium for 3 days then stimulated with TGF-β1 (1 ng/ml) for 24 hours. TGF-β1-inducible CAT activity in E box mutant construct transfectants was significantly reduced (>80%) compared to similarly-treated cultures expressing the wild-type expression vector (**E**) although an exogenous PAI-1 promoter-driven PAI-1-GFP imaging chimera (**F**) as well as the endogenous PAI-1 gene (both assessed as controls) were fully responsive to TGF-β1 stimulation (**G,H**).

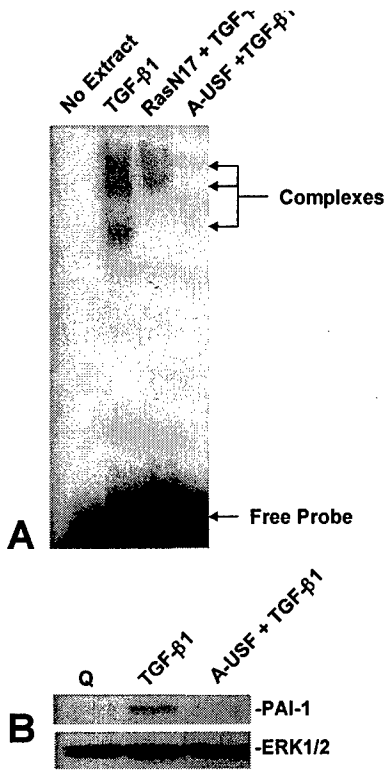

Figure 3. Dominant-negative USF inhibits formation of PAI-1 probe/nuclear factor complexes and attenuates PAI-1 expression in response to TGF-β1. T2 cells were transfected with the dominant-negative USF (A-USF) or Ras^{N17} constructs, cultured under quiescence conditions then stimulated with 1 ng/ml TGF-β1 for 3 hours prior to extraction of nuclear proteins for electrophoretic mobility shift assay (**A**) or for 5 hours for western analysis of cellular PAI-1 protein (**B**). Nuclear extracts from Ras^{N17}-transfectants failed to form the fastest migrating complex upon incubation with the ^{32}P-labeled 18-mer PAI-1 construct (**Figure 1A**). A-USF transfection not only abolished formation of all complexes (**A**) but also significantly attenuated (by >90%) TGF-β1-induced PAI-1 levels to that approximating quiescent (Q) controls (**B**).

Revised Statement of Work for Task 1

Task 1. To assess the effect of retroviral delivery of PAI-1 antisense expression vectors on the Angiogenic response to implanted human breast and PDVA carcinoma cells.

a. Confirm vector transduction, PAI-1 antisense transcript expression and down-regulated PAI-1 protein levels in the developing tumor vasculature upon co-implantation of MDA-MB-231 human breast (and PDVA) carcinoma cells and retroviral packaging cells into the mammary fat pad of immunodeficient nude (or syngeneic C57BL/6) mice.

b. Assess the ability of constitutive and inducible PAI-1 antisense transcript expression to disrupt the development and maintenance, respectively, of a tumor-dependent angiogenic response and the consequences of PAI-1 expression attenuation on tumor growth.

To be completed during the year 04 extension period.

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